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The use of polyacrylamide hydrogels to study the effects of matrix stiffness on nuclear envelope properties

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Running Head: Culture Conditions on Nuclear Envelope Composition

i. Summary

Matrix-derived mechanical cues influence cell proliferation, motility and differentiation. Recent findings clearly demonstrate that the nuclear envelope (NE) adapts and remodels in response to mechanical signals, including matrix stiffness, yet a plethora of studies have been performed on tissue culture plastic or glass that have a similar stiffness to cortical bone. Using methods that allow modulation of matrix stiffness will provide further insight into the role of the NE in physiological conditions and the impact of changes in stiffness observed during ageing and disease on cellular function. In this chapter, we describe the polyacrylamide hydrogel system, that allows fabrication of hydrogels with variable stiffness to better mimic the environment experienced by cells in most tissues of the body.

ii. Key Words

Mechanotransduction, hydrogels, extracellular matrix and stiffness

1.Introduction

Signals derived from the extracellular microenvironment regulate many cellular processes including proliferation, migration and differentiation. Our understanding of how cells sense and transmit these signals has increased rapidly in recent years and we are now beginning to appreciate the role of biophysical signalling, in addition to biochemical pathways that regulate these processes. Matrix stiffness has emerged as a major regulator of cellular behaviour and the stiffness of the microenvironment transmits 'outside-in' forces to cells. This process is dependent on ECM adhesions that convey force between the ECM and cytoskeleton[1]. Cells respond to outside-in signals by exerting actomyosin-based contractile forces on the matrix (inside-out forces) that increase cell stiffness and scale with ECM stiffness[2]. Rho/ROCK signalling is rapidly activated at ECM adhesions in response to matrix stiffness to augment actomyosin activity, via actin polymerisation and myosin light chain phosphorylation, and increase cell stiffness[3,4].

The nuclear envelope (NE) consists of an outer nuclear membrane (ONM) and an inner nuclear membrane (INM) with both membranes joined at nuclear pores[5]. Importantly, the NE has emerged as a major regulator of cytoskeletal organisation and differentiation[6]. Underlying the INM is an intermediate filament (IF) protein meshwork, called the nuclear lamina, which is composed of lamins (A/C and B) and lamina associated proteins[5]. The nuclear lamina is indirectly coupled to the cytoskeleton via the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex that spans the NE[7,8]. This complex is comprised of nesprin and SUN family members. Giant nesprin-1/2 isoforms reside on the ONM and bind F-actin via a pair of N-terminal Calponin Homology (CH) domains[9]. Complex stability is maintained

via interactions between the C-terminal Klarsicht, Anc-1, Syne-1 Homology (KASH) domain of the nesprins and the SUN domain of SUN1 and SUN2 in the perinuclear space[7,8]. SUN1/2 span the INM and interact directly with lamins A/C, physically coupling the actin cytoskeleton to the nuclear lamina[8]. Thus, the plasma membrane, actin cytoskeleton and nucleus function as a single mechanically coupled system. Recent studies have highlighted the importance of this network in matrix-derived mechanosignalling and lamin A levels scale with matrix stiffness in mesenchymal stem cells (MSCs)[10,11]. Lamin A also modulates actin dynamics to control the nuclear availability of the serum response factor (SRF)-coactivator myocardin-related transcription factor-A (MRTF-A) to regulate SRF mediated transcription[12]. Importantly, the LINC complex has recently been shown to directly transmit biophysical mechanical signals across the NE and mechanical stimulation of nesprin-1 giant on the ONM increases lamin A coupling to the INM[13].

The Young's modulus, E (a measure of material stiffness), of different tissues within the body demonstrates considerable variation, from very soft (fat 0.1-1kPa), to stiff (muscle 10-20kPa), to extremely stiff (cortical bone 10-20GPa)[14]. Moreover, ageing and disease augment tissue stiffness. For example, healthy aorta has a Young's modulus of between 10-20kPa, whereas atherosclerotic plaques contain a stiffened fibrous cap ($E=60-250\text{kPa}$) and aortas from spontaneously hypertensive rats display a 2-4 fold increase in stiffness compared to age-matched controls[15-18]. A plethora of studies have been performed on tissue culture plastic ($E=10\text{GPa}$) and glass ($E=55\text{GPa}$) that possess a similar Young's modulus to cortical bone ($E=10-20\text{GPa}$). However, following recent advances in our understanding of how the NE adapts and remodels in response to matrix-derived mechanical cues, methods that allow manipulation of these signals will provide a better understanding of NE

function in physiological and pathological conditions. One strategy to regulate ECM stiffness is via the utilisation of hydrogels that possess defined Young's moduli. Polyacrylamide (PA) hydrogels have a number of advantages including: 1) no specialised equipment is required for fabrication, 2) they possess constant surface chemistry despite altered mechanical properties and 3) only ECM molecules covalently linked to the hydrogel can serve as ligands for cell attachment[14,19]. In this chapter, we will discuss the process of PA hydrogel fabrication, a multistep process that includes coverslip activation, hydrogel polymerisation and hydrogel functionalisation.

2.Materials

(3-aminopropyl)-triethoxysilane (APES)

0.5% glutaraldehyde

Ammonium persulphate (APS), 10% dissolved in water

N,N,N,N' tetramethyl-ethylenediamine (TEMED)

Sulfo-SANPH, 1mg/ml in water

2.5% bis-acrylamide

40% acrylamide

Phosphate buffered saline (PBS)

Earle's balanced salt solution

30mm thickness No 1 coverslip

1mm thick glass slide

6-well culture plate

Collagen-1 (or ECM component of choice)

A trans-illuminator or UV lamp that irradiates at 300-460nm. We use a 25W UVP trans-illuminator.

3.Method

All steps are performed at room temperature, unless otherwise stated.

3.1 Coverslip activation

1. In a fume hood, cover the top surface of a 30mm coverslip with APES for 5 min.
2. Rinse 3 times with 2 mL distilled water, making sure that the top and bottom surfaces of the coverslip are well washed (see**Note 1**).
3. Immerse coverslips in 0.5% glutaraldehyde solution and incubate for 30-60 min.
4. Rinse 3 times with distilled water and air dry. In our hands, activated coverslips remain functional for 1 week and can be stored at room temperature.

3.2 Hydrogel preparation

1. Mix together the appropriate ratios of acrylamide and bis-acrylamide in distilled water or PBS. Typical ratios that we use, and their corresponding Young's moduli (determined by atomic force microscopy), are shown in Table 1 (see**Note 2**). The Young's moduli of other acrylamide/bis-acrylamide ratios can be found elsewhere[14] (see**Note3**).

2. To initiate hydrogel polymerisation, add 1:100 APS and 1:1000 TEMED to the aliquot of stock solution and mix well (see**Note 4**).
3. Place 50µl of the mix in the centre of a glass slide. Place the activated side of the coverslip into the solution, gently lowering the coverslip and being careful not to create air bubbles. Incubate for 5-10 min, using any leftover mix to assess polymerisation state (see**Note 5**).
4. Remove the glass slide and place the coverslip, hydrogel side up, into the bottom of a 6-well plate or 35mm petri dish (see**Note 6**).
5. Wash hydrogels with distilled water to remove any unpolymerised mixture. Hydrogels can be stored in distilled water or PBS at 4°C for 1 week.

3.3 Functionalising hydrogels for tissue culture

Cell attachment to the hydrogels requires an ECM coating. We use collagen, however, you can use any ECM component, depending on cell type and integrin pathways of interest. The ECM component is covalently cross-linked to the hydrogel and to achieve this we use sulfo-SANPH, a photo-activated protein cross-linker using the following method.

1. Remove the distilled water/PBS from the hydrogel.
2. Add 500-1000 µl of sulfo-SANPH, making sure the hydrogel is fully immersed to allow complete coverage (see**Note 7**).
3. Expose to 365nm UV light for 5-10 min. The hydrogel will be coated in dark red sulfo-SANPH. Repeat if necessary (see**Note 8**).

4. Remove the sulfo-SANPH and wash the hydrogel 3x in sterile PBS (see**note 9**).
5. Immerse the coverslip in 0.1-0.3mg/ml collagen or ECM component of your choice. Incubate at 4°C for between 1-4 hours (see**Note 10**).
6. Wash once in PBS and then incubate in prewarmed Earle's balanced salt solution (or equivalent) for 10-30 min at 37°C to allow the hydrogel to warm up (see**Note 11**).
7. Plate cells onto the hydrogel in their standard medium (see**Note 12**). A schematic of the final functionalised hydrogel set-up is shown in Figure 1.
8. Cells can be trypsinised and maintained on hydrogels for weeks.

4. Notes

1. It is important to wash well as any remaining APES will react with glutaraldehyde in subsequent steps leaving a brown material that could interfere with downstream experiments.
2. For mapping of cellular traction forces, fluorescent beads may be added to the hydrogel.
3. We use hydrogels that mimic the physiological and pathological stiffness experienced by VSMCs and fibroblasts. The choice of hydrogel stiffness is cell type dependent and should approximate conditions experienced *in vivo*.
4. For consistency between batches of hydrogels, we prepare a 50ml stock solution and perform atomic force microscopy (AFM) to confirm the Young's modulus of each stock. Solutions can be stored at 4°C for several months.

5. Extended polymerisation times can make it difficult to remove the coverslip from the slide.
6. Trying to lift the coverslip directly off the slide often results in snapping of the coverslip. To overcome this, we first slide the coverslip and then lift.
7. As sulfo-SANPH is light sensitive it should be made fresh and kept in the dark until required.
8. UV exposure makes the sulfo-SANPH change to a dark red colour. If this does not happen, then cross-linking has failed and the step needs to be repeated.
9. Perform all subsequent steps in a tissue culture hood to limit the chance of contamination.
10. We incubate for 1 hour but this may need to be increased, depending on cell type. The ECM coating will contribute to the overall stiffness of the functionalised hydrogel so it is important to keep the concentration of ECM component and the incubation time consistent. It is also important to confirm the functionalised hydrogel stiffness to confirm the ECM coat is not altering overall stiffness.
11. We find warming the hydrogel yields better cell attachment/viability.
12. Seeding number is cell size and assay dependent. For example, we aim for a confluency of between 60-70% for Western blotting whereas for immunofluorescence we aim for 20-30% confluency so that the cells are not touching one another. In our hands, confluent vascular smooth muscle cell (VSMC) and fibroblast cultures detach from the hydrogel.

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Table 1

Hydrogel stiffness (kPa)	Acrylamide (final %)	Bis-acrylamide (final %)
1.879 +/- 0.2835	5	0.1
11.78 +/- 1.270	7.5	0.15

Figure legend

Figure 1. Schematic diagram representing the final functionalised hydrogel set-up with cells attached.

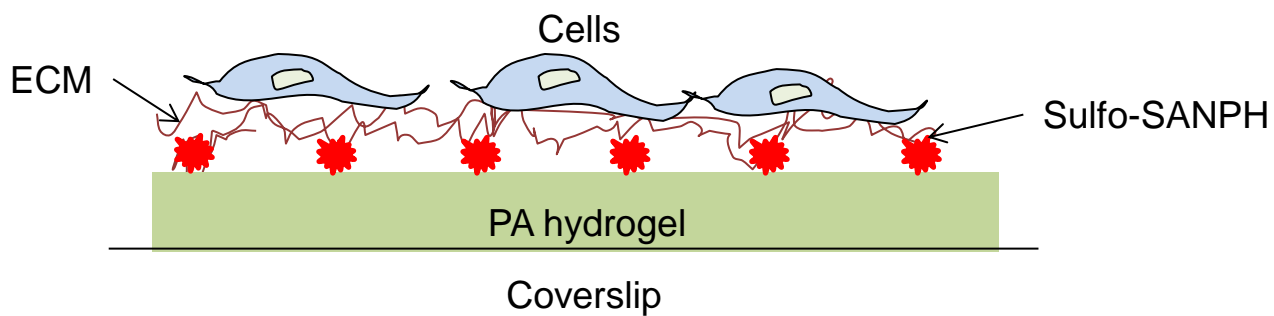


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